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14. ABSTRACT

Class IA PI3K p110 catalytic subunits are activated upon SH2 domain mediated binding of p85 regulatory subunits to tyrosine phosphorylated receptor tyrosine kinases (RTKs) or adaptor proteins. This activation can be enhanced by Ras, and is amplified by PTEN loss in the majority of advanced prostate cancers (PCa). We found that RTK inhibitors lapatinib and sorafenib could suppress PI3K in PTEN deficient PCa cells. However, analysis of p85 associated proteins by immunoblotting and LC/MS/MS failed to detect SH2 domain mediated interactions, indicating that these inhibitors were functioning downstream of PI3K. Significantly, p85 was associated primarily with p110β, indicating that PTEN loss may select for increased p110β expression due to basal RTK independent activity or activation by other mechanisms. Further studies to test this hypothesis are underway.

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prostate cancer, PI3 kinase, receptor tyrosine kinase

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INTRODUCTION

PI3 kinase pathway activation is common in advanced prostate cancer (PCa) and is mediated primarily by PTEN loss, suggesting that it may be independent of activation by upstream receptor tyrosine kinases (RTKs) or nonreceptor tyrosine kinases and therefore unresponsive to tyrosine kinase inhibitors. However, in studies presented in the proposal, we found that the p85 regulatory subunit of PI3 kinase is associated constitutively with ErbB3 and two other tyrosine phosphorylated proteins in PTEN deficient LNCaP and C4-2 PCa cells. Treatment with an ErbB2 inhibitor (lapatinib) did not rapidly decrease PI3 kinase activity, but combined treatment with lapatinib and sorafenib (a multi-kinase inhibitor) was as effective as a direct PI3 kinase antagonist (LY294002) at blocking PI3 kinase activity. Based on these data, we hypothesized that a small number of upstream RTKs (or nonreceptor tyrosine kinases) may be critical for PI3 kinase activation in PTEN deficient PCa, and that targeting these tyrosine kinases may be an effective approach for suppressing PI3 kinase activity and PCa growth *in vivo*. The objective of this proposal was to test these hypotheses, and more generally determine the molecular basis for basal PI3 kinase activity in PTEN deficient PCa cells. The specific aims were as follows:

Aim 1. Identify the p85 associated tyrosine phosphorylated proteins in PTEN deficient LNCaP and C4-2 PCa cells lines, and determine whether they mediate PI3 kinase activation.

Aim 2. Test the hypothesis that receptor tyrosine kinase inhibitors can be used to block p85 membrane recruitment and suppress PI3 kinase activity *in vivo* in PCa xenografts.

BODY

Aim 1. Identify the p85 associated tyrosine phosphorylated proteins in PTEN deficient LNCaP and C4-2 PCa cells lines, and determine whether they mediate PI3 kinase activation. We found previously a series of p85 associated proteins in LNCaP and C4-2 cells, and also showed that p85 was associated with ErbB3. Therefore, in the studies below we initially focused on whether ErbB3 was one of the detected p85 associated tyrosine phosphorylated proteins, and its role in PI3 kinase activation.

p85 interaction with ErbB3 is independent of ErbB3 phosphorylation and ErbB2 activity. We found previously that p85 was associated with ErbB3 (Fig. 1A), but it was not clear if ErbB3 was one of the major tyrosine phosphorylated proteins associated with p85 or whether it was mediating or contributing to PI3 kinase activation. Therefore, we further examined whether the p85 interaction with ErbB3 was dependent on ErbB3 phosphorylation and mediating PI3 kinase activation**.** To test this hypothesis, we determined whether initially depleting ErbB3 by immunoprecipitation with anti-ErbB3 would decrease the p85 associated ~190 kDa tyrosine phosphorylated band in a subsequent anti-p85 immunoprecipitation. As shown in figure 1B, ErbB3 could be substantially depleted from the lysate by an initial immunoprecipitation with anti-ErbB3. The ErbB-3 depletion also markedly decreased the amount of ErbB3 that was coprecipitated with p85. However, this ErbB-3 depletion did not decrease the intensity of the tyrosine phosphorylated band at ~190 kDa or other bands that were coprecipitated by anti-p85. Moreover, the pTyr blot further indicated that ErbB3 immunoprecipitated by the anti-ErbB3 Ab was not substantially tyrosine phosphorylated.

This result indicated that ErbB3 was not one of the major the tyrosine phosphorylated proteins associated with p85. However, we could not rule out the possibility that a small pool of heavily tyrosine phosphorylated ErbB3 was associated with p85, and was not cleared by the anti-ErbB3. Therefore, we next used siRNA to downregulate ErbB3 expression. As shown in figure 1C, total ErbB3 expression was markedly reduced by ErbB3 siRNA versus a control siRNA. Moreover, p85 associated ErbB3 was also decreased, although it again appeared that this decrease was less marked than the decrease in total ErbB3. Importantly, there was again no decrease in the p85 associated tyrosine phosphorylated protein at ~190 kDa (Fig. 1D). Moreover, there was no evident effect of the ErbB3 siRNA on PI3 kinase activity, as assessed by Akt phosphorylation at S473 or T308 (Fig. 1C).

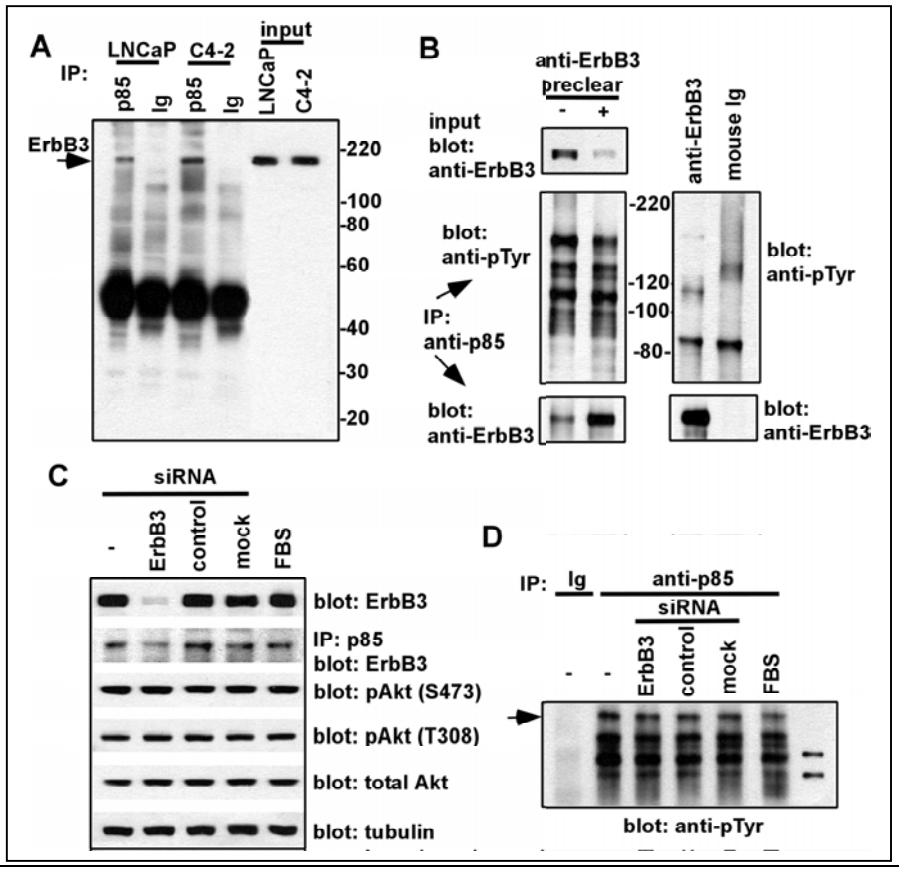

Figure 1. p85 interaction with ErbB-3 is not dependent on tyrosine phosphorylation in LNCaP cells. A, Lysates from serum starved LNCaP or C4-2 cells (2 days) were precipitated with anti-p85 (p85) or control rabbit IgG (Rab IgG), followed by blotting for ErbB-3. Input is 1% of the material used for the precipitation. B, LNCaP cells maintained in medium with 10% FBS were lysed in TBS buffer with 1% TX-100 and immunoprecipitated with anti-ErbB-3 and anti-p85 sequentially or with normal mouse serum (NMS) as control. The immunoprecipitates were immunoblotted with anti-p-Tyr or anti-ErbB-3. C and D, LNCaP cells transfected with siRNA of ErbB-3 or non-targeted control siRNA were maintained in RPMI-1640 with 10% FBS for 24 hr followed by serum starvation for 48 hr. Cell lysates were immunoprecipitated with anti-p85 followed by immunoblotting for anti-ErbB-3 (C) or anti-p-Tyr (D). Meanwhile, whole cell lysates (10μg) were subjected to immunoblotting for anti-pAkt to assess the PI3 kinase activity (C). Molecular markers are indicated at the margins.

Taken together, these results demonstrated that p85 was constitutively associated with ErbB3 in LNCaP cells, but indicated that ErbB3 was not one of the major tyrosine phosphorylated proteins associated with p85. To further address whether ErbB3 phosphorylation made any contribution to the p85-ErbB3 interaction, we treated LNCaP cells with a dual EGFR/ErbB2 inhibitor (lapatinib) to suppress any basal tyrosine phosphorylation of ErbB3. Using an anti-pTyr immunoprecipitation followed by immunoblotting to detect tyrosine phosphorylated proteins, we found that lapatinib suppressed the basal tyrosine phosphorylation of both EGFR and ErbB2 (Fig. 2A). In contrast, there was no detectable tyrosine phosphorylation of ErbB3 in the presence or absence of lapatinib (10μM for 6 hours), and no effect of lapatinib on Akt phosphorylation. Moreover, lapatinib did not decrease the interaction between p85 and ErbB3, strongly supporting the conclusion that this interaction is independent of ErbB3 phosphorylation (Fig. 2B).

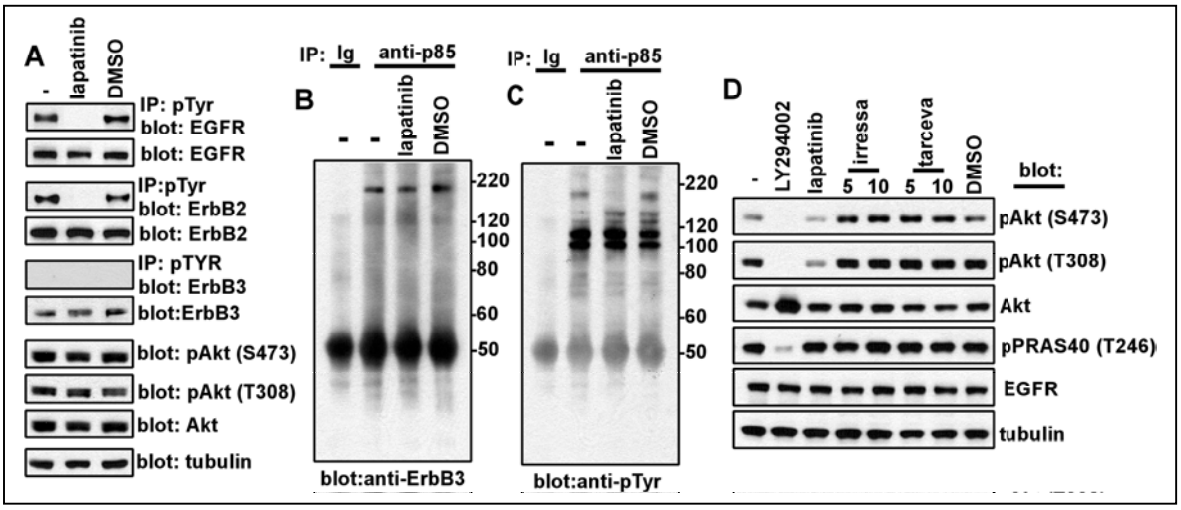

Figure 2. ErbB-2 inhibition suppresses PI3 kinase activity in LNCaP cells independently of tyrosine phosphorylation of ErbB-3. A-C, serum starved LNCaP cells (2 days) were treated with EGFR/ErbB-2 dual inhibitor lapatinib or vehicle (DMSO) for 6 hr at the concentration of 10μM. A, cell lysates were immunoprecipitated with anti-p-Tyr followed by immunoblotting for anti-EGFR, ErbB-2, or ErbB-3. Phosphorylation of Akt was also assessed. B and C, cell lysates were immunoprecipitated with anti-p85 or rabbit IgG followed by immunoblotting for anti-ErbB-3 (B) or anti-p-Tyr (C). D, serum starved LNCaP cells (2 days) were treated with PI3 kinase inhibitor LY294002 (20μM) for 2 hr, lapatinib (5μM) for 24 hr, EGFR inhibitors Iressa (5 or 10μM) or Tarceva (5 or 10μM) for 24 hr. Cell lysates (10μg) were subjected to immunoblotting analysis for PI3 kinase activity. Molecular markers are indicated at the margins for panels B and C.

Surprisingly, the intensity of the p85 associated band at ~190 kDa detected by pTyr immunoblotting was selectively decreased by lapatinib, suggesting that it may be an EGFR or ErbB2 substrate (Fig. 2C). Based on this observation, we further examined the effects of longer exposure to lapatinib on PI3 kinase activity. After 24 hours, lapatinib at 5 μM decreased Akt phosphoryation, although phosphorylation of an Akt substrate (PRAS40) was not effected (Fig. 2D). In contrast to these effects of lapatinib, Akt phosphorylation was not decreased by two EGFR specific inhibitors (Irressa and Tarceva), indicating that the effects of lapatinib were mediated through inhibition of ErbB2.

P85 association with tyrosine phosphorylated proteins is not mediated by direct p85 SH2 domain binding. The p85 subunits associate with tyrosine phosphorylated proteins primarily through their SH2 domains, which bind to proteins bearing pYxxM motifs. To determine whether constitutive tyrosine phosphorylation of a specific protein was mediating p85 recruitment directly through SH2 domain binding, we next immunoblotted the p85 immunoprecipitates with a pYxxM motif specific antibody. This antibody weakly detected several discrete p85 associated proteins between 130-200 kDa in lysates from LNCaP cells grown in 10% FBS (Fig. 3B). Discrete major p85 associated bands at ~190 kDa were detected by the anti-pYxxM antibody after EGF or heregulin-β1 stimulation, with the band after heregulin-β1 being consistent with ErbB3 (which contains 6 pYxxM motifs). In contrast, the pYxxM antibody did not detect discrete p85 associated proteins, or proteins corresponding to those found by pTyr blotting, in lysates from cells cultured in serum free medium (Fig. 3B). As a further sensitive assay to determine whether proteins containing pYxxM motifs were present in serum starved LNCaP cells, whole cell lysates were immunoprecipitated with an anti-pTyr Ab and then immunoblotted with the pYxxM motif Ab. As shown in figure 3C, several bands could be detected when cells were grown in 10% FBS or were stimulated with EGF or heregulin-β1, but not in the serum starved cells. Taken together, these data indicated that the association between p85 and tyrosine phosphorylated proteins was not mediated by direct p85 SH2 domain binding.

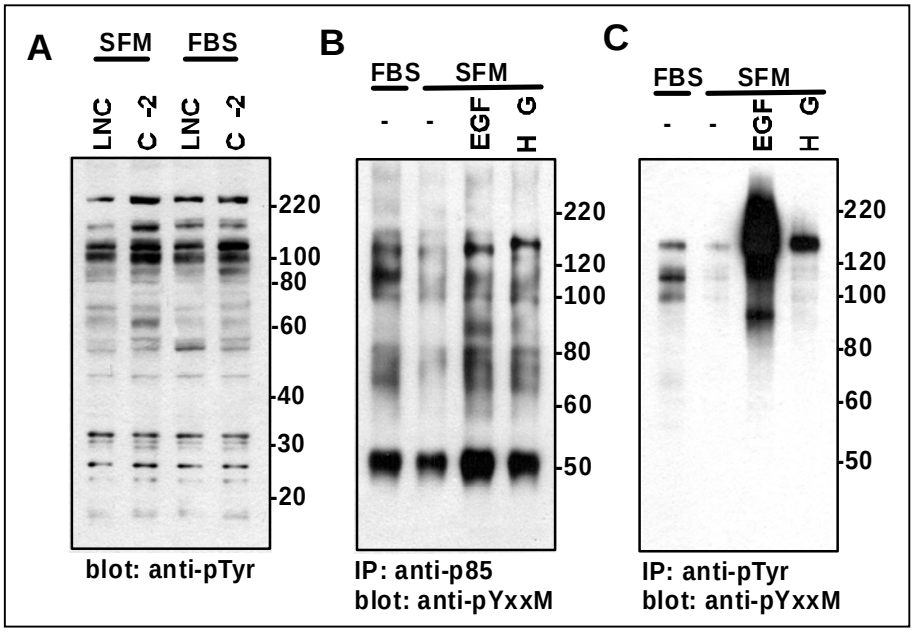

Figure 3. p85 association with tyrosine phosphorylated proteins in serum starved PTEN deficient cells LNCaP cells is not mediated by the p85 SH2 domain. A, cell lysates (10 g) from LNCaP or C4-2 cells grown in serum free medium (SFM) as well as in medium with 10% FBS were immunoblotted for anti-p-Tyr to assess tyrosine phosphorylated proteins. B and C, LNCaP cells were either maintained in medium with 10%FBS or serum starved for 2 days. Serum starved cells were then stimulated with EGF (20ng/ml, 5 min) or HRG-β1 (40ng/ml, 15 min). Cell lysates were immunoprecipitated with anti-p85 (B) or anti-p-Tyr (C) and the immunoprecipitates were blotted with anti-pYXXM. Molecular markers are indicated at the margins.

PI3K is not associated with tyrosine phosphorylated proteins in PC3 cells. The data above in conjunction with our previopus data indicated that ErbB2, in conjunction with one or more kinases targeted by sorafenib, contributed to PI3K pathway activation in PTEN deficient LNCaP cells. Moreover, this inhibition correlated with loss of tyrosine phosphorylated p85 associated proteins. However, we could not conclude from these results whether these tyrosine phosphorylated proteins were required for p85 membrane recruitment and PI3K activation. Therefore, to further assess the possible importance of p85 membrane recruitment by tyrosine phosphorylated proteins, we examined p85 associated proteins from PC3 cells (also a PTEN deficient PCa cell line) and a series of other cell lines. Significantly, the only tyrosine phosphorylated band associated with p85 in PC3 cells was ~110 kDa, consistent with the p110 catalytic subunit of PI3K (Fig. 4A). This band was also found in a subset of other cell lines, with no other tyrosine phosphorylated bands being common to multiple cells.

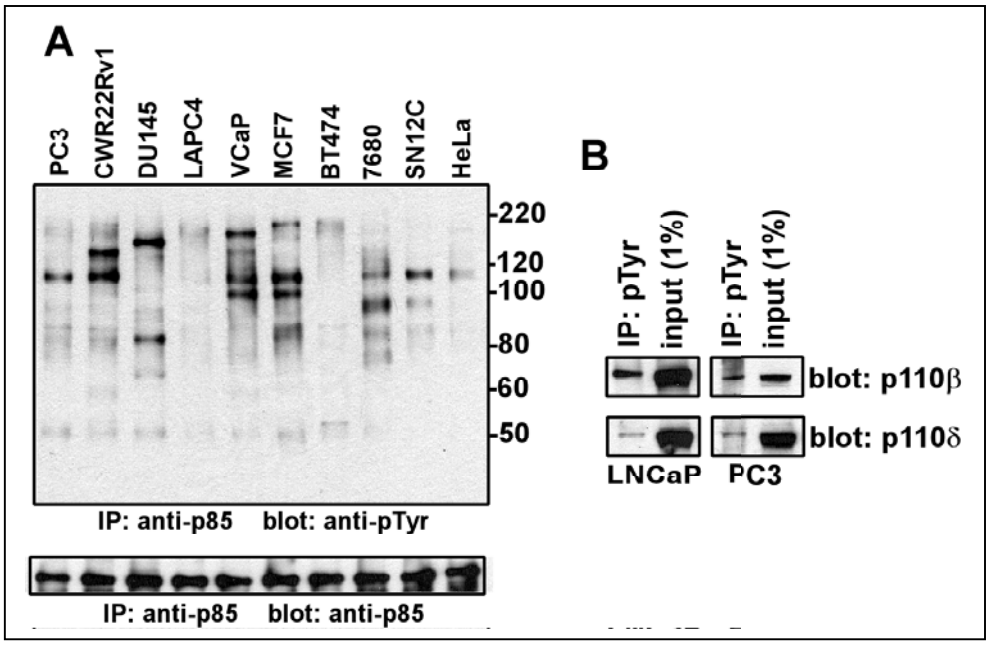

Figure 4. p85 is not associated with multiple tyrosine phosphorylated proteins in PC-3 cells. A, human prostate cancer cell lines PC-3, 22Rv1, Du145, LAPC4, VCaP, human breast cancer lines MCF-7, BT474, human renal carcinoma cell lines 786O (PTEN deficient), SN12C, and human cervix caner cell line Hela were serum starved for 1 day. Cell lysates were immunoprecipitated with anti-p85 and the immunoprecipitates were immunoblotted for anti-p-Tyr or anti-p85. Molecular markers are indicated at the left margin. B, Serum starved LNCaP (2 days) and PC-3 cells (1 day) were lysed and immunoprecipitated with anti-p-Tyr followed by immunoblotting for anti-p110β or δ. Input is 1% of the material used for the precipitation.

To support the conclusion that the tyrosine phosphorylated protein at ~110 kDa was the PI3K catalytic subunit, we carried out anti-pTyr immunoprecipitations followed by immunoblotting for each of the p110 isoforms. As shown in figure 4B, total p110 levels were higher in LNCaP versus PC3 cells (inputs), but comparable levels were precipitated by the anti-pTyr antibody (with this blot indicating that ~1% of p110β in serum starved PC3 cells is tyrosine phosphorylated). It should be noted that p110 in these experiments may be precipitated indirectly through an association with another tyrosine phosphorylated protein such as p85 in LNCaP cells,

although the p110 precipitation from PC3 is more likely direct as there is no detectable tyrosine phosphorylation of p85 in these cells (Fig. 4A). In contrast to p110β, expression of p110δ is more comparable in LNCaP and PC3 cells, and only a very small fraction appears to be tyrosine phosphorylated. Finally, there was no detectable tyrosine phosphorylation of p110α in either cell (data not shown).

ErbB2 inhibition suppresses PI3K activity in PC3 cells. The above results indicated that basal PI3K activity in PC3 cells was not dependent on p85 recruitment by an activated RTK or tyrosine phosphorylated adaptor protein. Therefore, we next determined whether basal PI3K activity in serum starved PC3 cells was still inhibited by lapatinib or sorafenib. In contrast to LNCaP cells, lapatinib at 5-10 μM was able to rapidly suppress PI3K activity in serum starved PC3 cells (Fig. 5A) and in PC3 cells grown in 10% FBS (Fig. 5B). Sorafenib by itself was not clearly active, but could enhance the activity of lapatinib, supporting the conclusion that these drugs were inhibiting PI3K pathway activity by a mechanism distinct from preventing SH2 domain mediated recruitment of p85. As in LNCaP cells, the EGFR specific inhibitors (Irressa and Tarceva) were much less effective than lapatinib, indicating that the effects of lapatinib were due to ErbB2 inhibition (Fig. 5C). Consistent with this conclusion, EGFR downregulation by shRNA had no apparent effect on Akt phosphorylation or activity (as assessed by PRAS40 phosphorylation) in the PC3 cells (Fig. 5D).

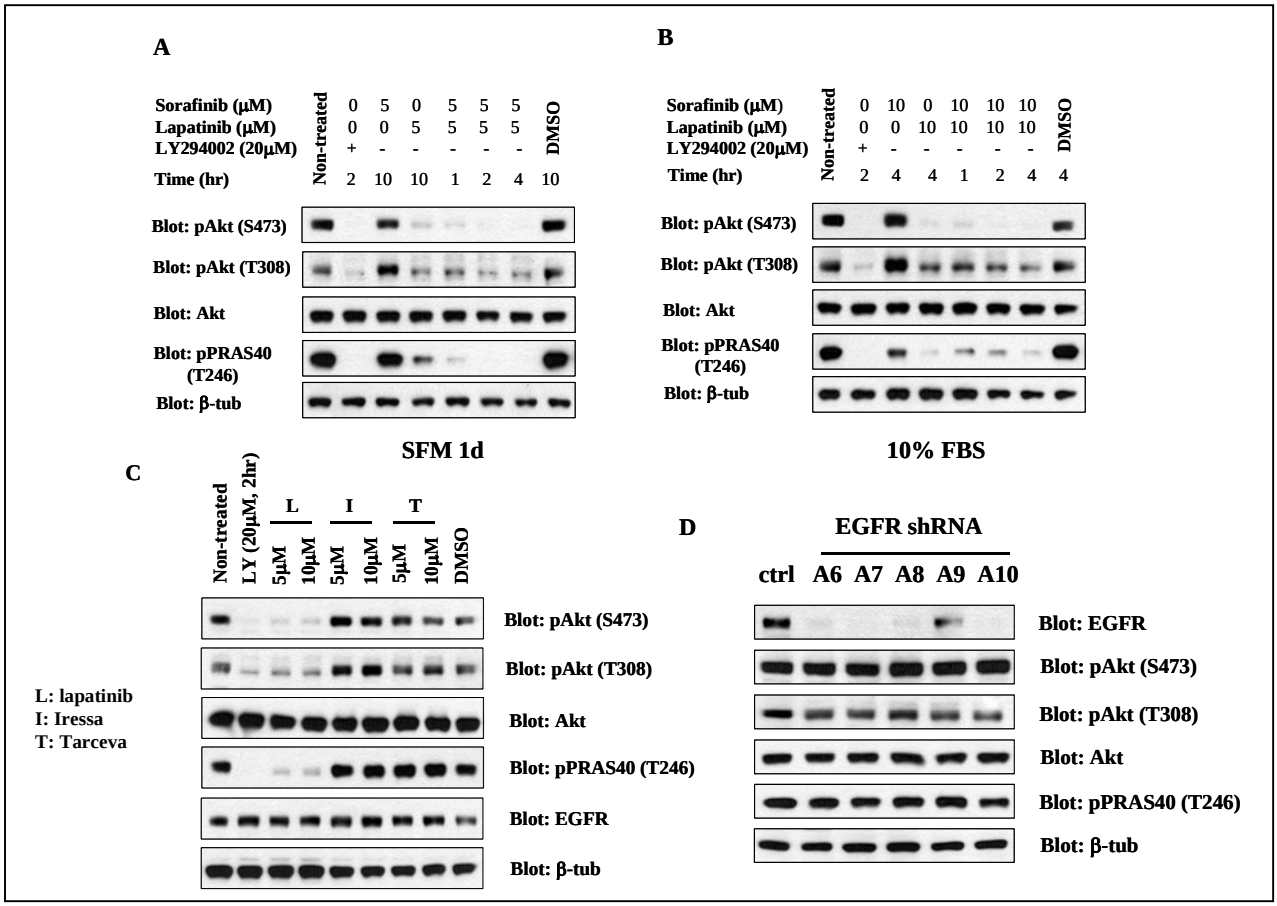

Figure 5. ErbB2 inhibition strongly suppresses PI3 kinase activity in PC-3 cells. A, PC-3 cells were serum starved for 1 day and then treated with sorafenib (5μM, 10 hr), lapatinib (5μM, 10 hr), or combination (5μM each) for 1, 2, or 4 hours. Whole cell lysates (10μg) were immnolotted with anti-pAkt or anti-pPRAS40 to assess PI3 kinase activity, B, PC-3 cells grown in medium with 10% FBS were treated with sorafenib (10μM, 4 hr), lapatinib (10μM, 4 hr), or combination (10μM each) for 1, 2 , or 4 hours, and cell lysates (10μg) were immnolotted for anti-pAkt or anti-pPRAS40. C, serum starved PC-3 cells (1 day) were treated with PI3 kinase inhibitor LY294002 (20μM) for 2 hr, lapatinib (5 or 10μM) for 24 hr, EGFR inhibitors Iressa (5 or 10μM) or Tarceva (5 or 10μM) for 24 hr. Cell lysates (10μg) were immnolotted with anti-pAkt or anti-pPRAS40. D, PC-3 cells were infected with virus containing EGFR shRNAs (A6, A7, A8, A9 or A10). Cells were maintained in DMEM medium with 10% FBS for 2 days and then serum starved for 1 day. Cell lysates (10μg) were immnolotted with anti-pAkt or anti-pPRAS40.

p110 tyrosine phosphoryation is mediated by c-Src and does not regulate PI3K activity. Although the functional significance of p110 tyrosine phosphorylation is not clear, based on these results we considered that lapatinib or sorafenib may be suppressing p110 catalytic activity by inhibiting its tyrosine phosphorylation. However, p85 immunoprecipitations followed by pTyr immunoblotting showed that neither lapatinib or sorafenib treatment, or the combination, decreased tyrosine phosphoryation of the p85 associated p110 band in PC3 cells (Fig. 6A). Similarly, pTyr immunoprecipitations followed by p110 immunoblotting indicated that p110β and p110δ tyrosine phosphorylation were not affected by lapatinib or sorafenib, or by the combination (Fig. 6B). Importantly, blotting for pAkt and pPRAS40 indicated that PI3K activity was decreased within 2 hours by the combined lapatinib plus sorafenib used in this experiment (Fig. 6B).

We next examined the Phosphosite and Scansite databases to determine previously identified sites of tyrosine phosphorylation on the PI3 kinase p110 catalytic subunits and candidate kinases for these sites, which suggested phosphorylation by c-Src family kinases. Strikingly, treatment with c-Src inhibitors (PP2 or dasatinib) resulted in the rapid and complete loss of p110 tyrosine phopshorylation in serum starved PC3 cells, as shown by p85 immunoprecipitation followed by pTyr blotting (Fig. 6A). Consistent with p110β being the predominant tyrosine phosphorylated p110 isoform in PC3 cells (see Fig. 5B), pTyr immunoprecipitation followed by p110 blotting showed a marked decline in p110β tyrosine phosphorylation (Fig. 6B). However, in contrast to the effects of sorafenib plus lapatinib, c-Src inhibition did not markedly suppress Akt phosphorylation or activity. It should again be noted that p110 may be precipitated by anti-pTyr through an association with another tyrosine phosphorylated protein. However, the coincident loss of the p85 associated tyrosine phosphorylated band at 110 (Fig. 6A) indicates that c-Src inhibitors are directly decreasing p110β and p110δ phosphorylation.

PP2 and dasatinib also markedly decreased both p110 and p110δ tyrosine phosphorylation in LNCaP cells, but again did not significantly decrease Akt phosphorylation or activity (Fig. 6C). Moreover, the tyrosine phosphoryated p85 associated bands at ~130-190 kDa in LNCaP cells were also markedly diminished by c-Src inhibition (Fig. 6D). The loss of these latter bands,

without an effect of PI3K pathway activity, further supports the conclusion that PI3K activity is not dependent on p85 recruitment by a tyrosine phosphorylated RTK or adaptor protein.

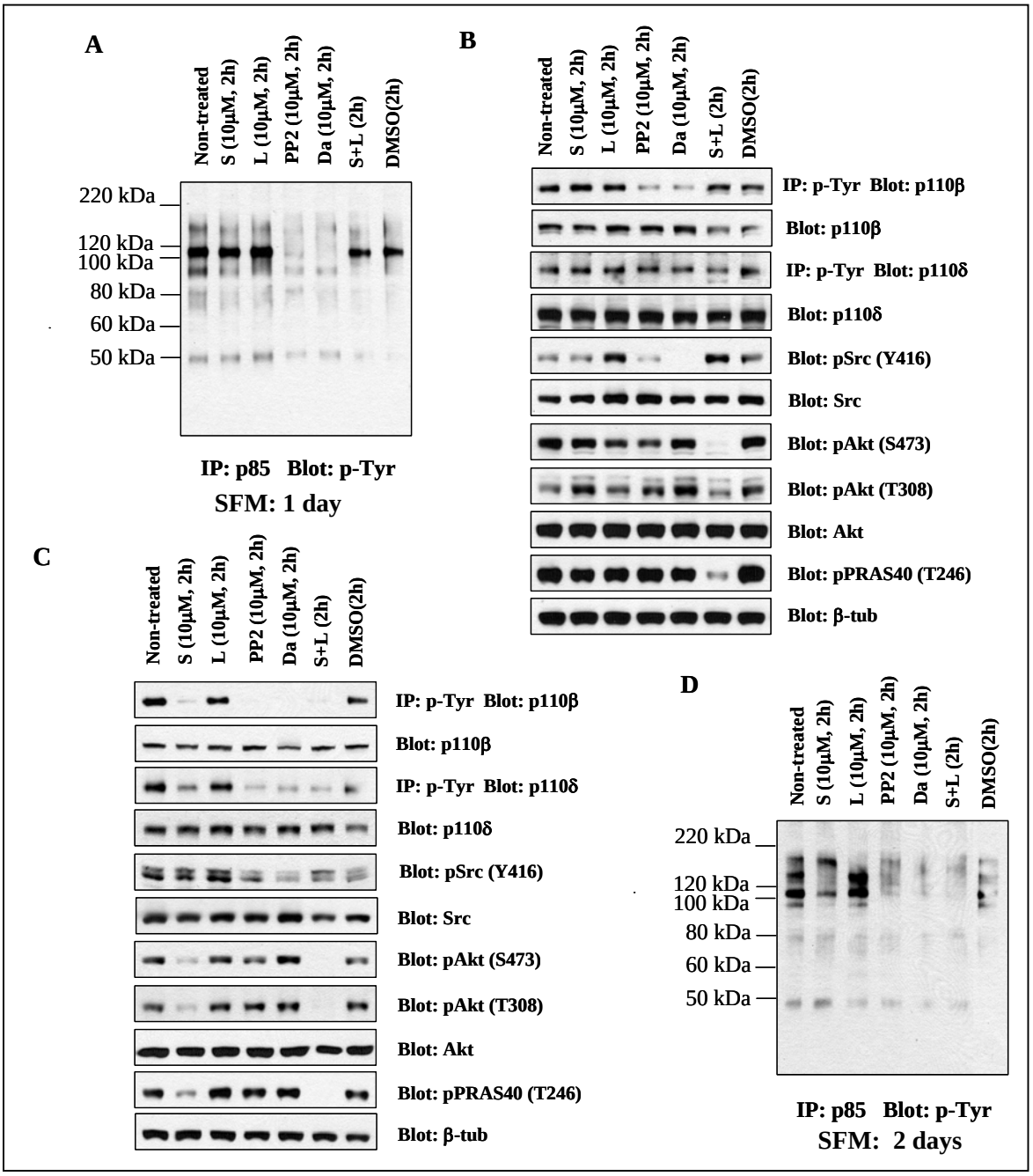

Figure 6. Phosphorylations of p110β and p110δ are mediated by c-Src but not correlated with PI3 kinase activity. A and B, serum starved PC-3 cells (1 day) were treated with sorafenib, lapatinib, combined sorafenib and lapatinib, Src inhibitors PP2 or dasatinib for 2 hr, all at 10μM. A, cell lysates (10μg) were immunoprecipitated with anti-p85 and the immunoprecipitates were immunoblotted for anti-p-Tyr. B, cell lysates were immunoprecipitated with anti-p-Tyr followed by immunoblotting for anti-p110β or δ. Whole cell lysates (10μg) were immunoblotted with

pSrc, pAkt, pPRAS40, total Src or Akt. C and D, serum starved LNCaP cells (2 days) were treated with sorafenib, lapatinib, combined sorafenib and lapatinib, PP2 or dasatinib for 2 hr, all at 10μM. B, cell lysates were immunoprecipitated with anti-p-Tyr followed by blotting for anti-p110β or δ. Whole cell lysates (10μg) were immunoblotted with pSrc, pAkt, pPRAS40, total Src or Akt. D, cell lysates were immunoprecipitated with anti-p85 and the immunoprecipitates were immunoblotted with anti-p-Tyr. Molecular markers are indicated at the left margins of panels A and D.

Aim 2. Test the hypothesis that receptor tyrosine kinase inhibitors can be used to block p85 membrane recruitment and suppress PI3 kinase activity *in vivo* in PCa xenografts.
Our focus over the past year has been on studies in Aim 1 to understand the molecular basis for PI3 kinase activation and PTEN deficient cells and the identity of upstream kinases, which can then be assessed in vivo. However, we have carried out a series of further in vitro studies to assess effects of EGFR and ErbB2 siganling pathways on PCa cells. These data have recently been published and are included in the Appendix (Cai et al., 2009). We are currently initiating in vivo studies with lapatinib in xenograft models and genetically modified mouse models. Moreover, we have developed and are initiating a phase I/II trial of lapatinib in combination with maximal androgen suppression in patients with advanced castration resistant PCa, which will provide \further critical insight into effects on PI3 kinase signaling in vivo over the next year.

KEY RESEARCH ACCOMPLISHMENTS

- Characterization of factors regulating basal PI3 kinase pathway activity in prostate cancer cell.

- Identification of mechanism of action for upstream kinases EGFR and ErbB2 in prostate cancer.

REPORTABLE OUTCOMES

Cai,C., Portnoy,D.C., Wang,H., Jiang,X., Chen,S., and Balk,S.P. (2009). Androgen receptor expression in prostate cancer cells is suppressed by activation of epidermal growth factor receptor and ErbB2. Cancer Res. 69, 5202-5209

CONCLUSION

We have examined whether basal PI3K activity in PTEN deficient PCa cells was dependent on p85 binding to particular tyrosine phosphorylated RTKs or adaptor proteins, which might then be therapeutic targets. Immunobotting of proteins associated with p85 in PTEN deficient LNCaP and C4-2 PCa cells (serum starved or grown in serum containing medium) showed discrete tyrosine phosphorylated proteins between ~130-190 kDa, but these proteins were not clearly recognized by an anti-pYxxM motif antibody. LC/MS/MS analysis showed that ErbB3 was associated with p85 in serum starved cells LNCaP cells, but subsequent studies indicated that ErbB3 was not one of the discrete tyrosine phosphorylated proteins associated with p85, and that this interaction was not dependent of ErbB3 tyrosine phosphoryation (see below). Moreover, the recovery of p85 associated tyrosine phosphorylated proteins was abrogated by c-Src inhibitors, without an effect on PI3K pathway activity. Finally, p85 was not associated with tyrosine phosphorylated proteins in PTEN deficient PC3 PCa cells. Taken together, these results showed

that PI3K activity in PTEN deficient PCa cells was not dependent on p85 SH2 domain mediated binding to tyrosine phosphorylated RTKs or adaptor proteins.

We could not consistently detect basal ErbB3 tyrosine phosphorylation in serum starved LNCaP cells, and immunodepletion and siRNA methods further indicated that ErbB3 was not one of the discrete p85 associated tyrosine phosphorylated proteins in LNCaP cells. Nonetheless, we used lapatinib (dual EGFR/ErbB2 inhibitor) to further determine whether suppressing any low levels of basal ErbB3 tyrosine phosphorylation would abrogate the p85 interaction with ErbB3. Surprisingly, lapatinib markedly decreased p85 association with a discrete tyrosine phosphorylated protein at ~190 kDa as detected by pTyr immunoblotting, but had no effect on p85 binding to ErbB3 (as determined by ErbB3 immunoblotting). The most straightforward interpretation of these results is that the ~190 kDa tyrosine phosphorylated protein associated with p85 protein is a direct or indirect target of EGFR/ErbB2 that is unrelated to ErbB3, although we cannot completely rule out the possibility that it reflects a small pool of highly tyrosine phosphoryated ErbB3. In any case, the data clearly show that most or all of the ErbB3 association with p85 is not dependent on ErbB3 phosphorylation and is presumably independent of the p85 SH2 domains, with the p85 SH3 domain binding to a proline rich region in ErbB3 providing an alternative basis for the association.

While lapatinib did not impair the p85:ErbB3 interaction, lapatinib treatment of LNCaP cells for 24 hours substantially suppressed PI3K activity as assessed by decreased Akt phosphorylation at S473 and T308. This inhibition by lapatinib alone was more dramatic in PC3 cells, and could be enhanced by the multi-kinase inhibitor sorafenib in both cells. Moreover, sorafenib alone at 10 M in serum starved LNCaP cells could rapidly suppress basal PI3 kinase activity. Interestingly, sorafenib treatment also markedly decreased the recovery of p85 associated tyrosine phosphorylated proteins at ~130 and ~150 kDa in LNCaP cells, which suggested that these proteins might be mediating p85 recruitment. However, as noted above, these proteins were not recognized by the pYxxM motif antibody and were not p85 interacting RTKs or adaptor proteins that are directly targeted by sorafenib. Moreover, they were not observed in PC3 cells, indicating they are not generally critical for basal PI3K activity. Finally, treatment of LNCaP cells with c-Src inhibitors resulted in loss of these bands in LNCaP cells without a loss in basal PI3K activity (see below), further indicating that they are not critical for p85 recruitment and PI3K activity.

An alternative mechanism by which RTK inhibitors could possibly be suppressing PI3K activity is by decreasing tyrosine phosphorylation of the p110 catalytic subunit. Previous studies have shown that p110 is tyrosine phosphoryated at multiple sites, although the functional importance of these sites and the relevant kinases remain to be determined. pTyr immunoprecipitation followed by immuoblotting with isoform specific p110 antibodies indicated that p110β, and to a lesser extent p110δ, were tyrosine phosphorylated in both serum starved LNCaP and PC3 cells. Sorafenib decreased both p110β and p110γ phosphorylation in LNCaP cells, coincident with its inhibition of PI3 kinase activity. However, p110 phosphorylation was not suppressed by lapatinib in either cell, or by the combination of lapatinib plus sorafenib in PC3 cells. In contrast, c-Src inhibition markedly decreased p110 tyrosine phosphorylation, but did not suppress PI3K pathway activity. Taken together, these results indicate that lapatinib and sorafenib are not suppressing PI3K activity by inhibiting p110 phosphorylation, and that p110 tyrosine phosphorylation is not critical for PI3K activity in PTEN deficient PCa cells. Overall, our

conclusions with respect to the RTK inhibitors is that their effects on PI3K pathway activity are not due to altered p85 recruitment, Ras binding (data not shown), or p110 phosphoprylation, but may reflect regulation of other PIP3 phosphatases or possibly protein phosphatases that target Akt or PDK1. Interestingly, the PIP3 phosphatase SHIP2 has been found to negatively regulate Akt phopshorylation in PC3 cells, although this was not observed in LNCaP cells.

While the p110α catalytic subunit is activated by mutation in multiple cancers, a recent study has shown that cellular transformation in the prostate specific PTEN-null mouse PCa model is dependent on the p110β isoform Similarly, PI3K activity and cell growth in several PTEN deficient cell lines, including LNCaP and PC3, have been found to be p110b dependent However, the molecular basis for this dependency on p110b for PI3K activity in PTEN deficient tumors has not been established. Consistent with these reports, we found that p85 was associated predominantly with p110β. Moreover, based on the findings in this study, we suggest that the basal RTK independent activity of p110β is markedly amplified by PTEN loss and drives the selection for increased expression of p110b (versus p110α) in PTEN deficient cells. An important corallary of this hypothesis is that p110β selective inhibitors may be effective initially or in early stage disease, but RTK activation in advanced tumors may allow cells to readily adapt by signaling through p110α.

REFERENCES

Cai,C., Portnoy,D.C., Wang,H., Jiang,X., Chen,S., and Balk,S.P. (2009). Androgen receptor expression in prostate cancer cells is suppressed by activation of epidermal growth factor receptor and ErbB2. Cancer Res. 69, 5202-5209

APPENDICES

One published manuscript.

Androgen Receptor Expression in Prostate Cancer Cells Is Suppressed by Activation of Epidermal Growth Factor Receptor and ErbB2

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Abstract

Prostate cancers (PCa) that relapse after androgen deprivation therapies [castration-resistant PCa (CRPC)] express high levels of androgen receptor (AR) and androgen-regulated genes, and evidence from several groups indicates that ErbB family receptor tyrosine kinases [epidermal growth factor (EGF) receptor (EGFR) and ErbB2] may contribute to enhancing this AR activity. We found that activation of these kinases with EGF and heregulin-β1 rapidly (within 8 hours) decreased expression of endogenous AR and androgen-regulated PSA in LNCaP PCa cells. AR expression was similarly decreased in LAPC4 and C4-2 cells, but not in the CWR22Rv1 PCa cell line. The rapid decrease in AR was not due to increased AR protein degradation and was not blocked by phosphatidylinositol 3-kinase (LY294002) or MEK (UO126) inhibitors. Significantly, AR mRNA levels in LNCaP cells were markedly decreased by EGF and heregulin-β1, and experiments with actinomycin D to block new mRNA synthesis showed that AR mRNA degradation was increased. AR mRNA levels were still markedly decreased by EGF and heregulin-β1 in LNCaP cells adapted to growth in androgen-depleted medium, although AR protein levels did not decline due to increased AR protein stability. These findings show that EGFR and ErbB2 can negatively regulate AR mRNA and may provide an approach to suppress AR expression in CRPC. [Cancer Res 2009;69(12):5202–9]

Introduction

Androgen receptor (AR) plays a central role in prostate cancer (PCa), with androgen deprivation therapies being the standard initial systemic treatment, but tumors eventually recur despite castrate androgen levels. These castration-resistant PCas (CRPC) express high levels of AR mRNA, AR protein, and androgen-regulated genes, indicating that AR transcriptional activity has been reactivated. One mechanism contributing to this reactivation is increased intratumoral androgen synthesis, but it seems clear that PCa adapts to androgen deprivation through multiple mechanisms that generate adequate AR activity despite castrate levels of circulating androgens (1–5). Evidence from several groups indicates that the ErbB family receptor tyrosine kinases ErbB1 [epidermal growth factor (EGF) receptor (EGFR)] and ErbB2 (HER2, Neu) contribute to enhancing AR activity in CRPC. Studies in PCa cell line and xenograft models have found increased EGFR or ErbB2 expression in tumors that relapse after castration, although this is not a consistent finding in patient samples and these receptors may also be enhanced by increased expression of ErbB ligands (6–14).

EGF can increase AR transactivation at low androgen levels, which may be mediated by increased expression or phosphorylation of the transcriptional coactivator protein TIF2/GRIP1 (15–18). The Ras-Raf-mitogen-activated protein kinase (MAPK) pathway and c-Src, which are activated downstream of EGFR, may also enhance AR responses to low levels of androgen (19–21). ErbB2 expression was increased in the LAPC4 xenograft model of CRPC, and a dual EGFR/ErbB2 inhibitor could reduce AR transcriptional activity and inhibit PCa xenograft growth after castration (6, 22). In CWR22 xenograft-derived CWR-R1 cells, heregulin stimulation of ErbB2 enhanced AR activity and cell growth (23). Other studies have shown that ErbB2 can enhance AR stability and that ErbB2 inhibition decreases AR DNA binding activity at low levels of androgen levels by a phosphatidylinositol 3-kinase (PI3K)–dependent, Akt-independent mechanism (6, 24, 25). In contrast, some studies indicate that ErbB2 enhances AR activity through the MAPK pathway or Akt (26, 27).

ErbB signaling also has been reported to negatively regulate AR expression and activity. In one study, EGF decreased AR mRNA and expression of androgen-regulated genes in LNCaP cells (28). In other studies, heparin binding EGF (HB-EGF) was found to decrease AR protein expression through activation of mTOR and decreased AR mRNA translation (29, 30). EGF also decreased PSA expression and secretion via the PI3K/Akt pathway in androgen-independent LNCaP-C81 cells (31). Finally, Akt in LNCaP cells may phosphorylate AR and enhance its ubiquitination by Mdm2 and degradation, but this seems to be dependent on cell passage number (32–36). Due to the significance of ErbB signaling in PCa, this study further examined how both EGF and heregulin-β1 regulate AR expression and activity in PCa cells.

Materials and Methods

Cell culture. LNCaP, LAPC4, C4-2, and CWR22-Rv1 cells were cultured in RPMI 1640/10% fetal bovine serum (FBS). HeLa and PC3-AR cells were cultured in DMEM/10% FBS. For DHT treatment, cells were grown to 50% to 60% confluence in medium with 5% charcoal/dextran-stripped serum (CSS; Hyclone) for 2 d before treatment.

Real-time reverse transcription–PCR. Primers and probes for quantitative real-time reverse transcription–PCR (RT-PCR) amplification were as follows: PSA forward, 5-GATGAAACAGGCTGTGCCG-3; PSA reverse, 5-CCTCACAGCTACCCACTGCA-3; PSA probe, 5-FAM-CAGGAACAAAAGCGTGATCTTGCTGGG-3; AR forward, 5′-GGAATTCCTGTGCATGAAA-3′; AR reverse, 5′-CGAAGTTCATCAAAGAATT-3′; AR probe, 5′-FAM-CTTCAGCATTATTCCAGTG-3′. Each reaction used 50 ng RNA and was normalized

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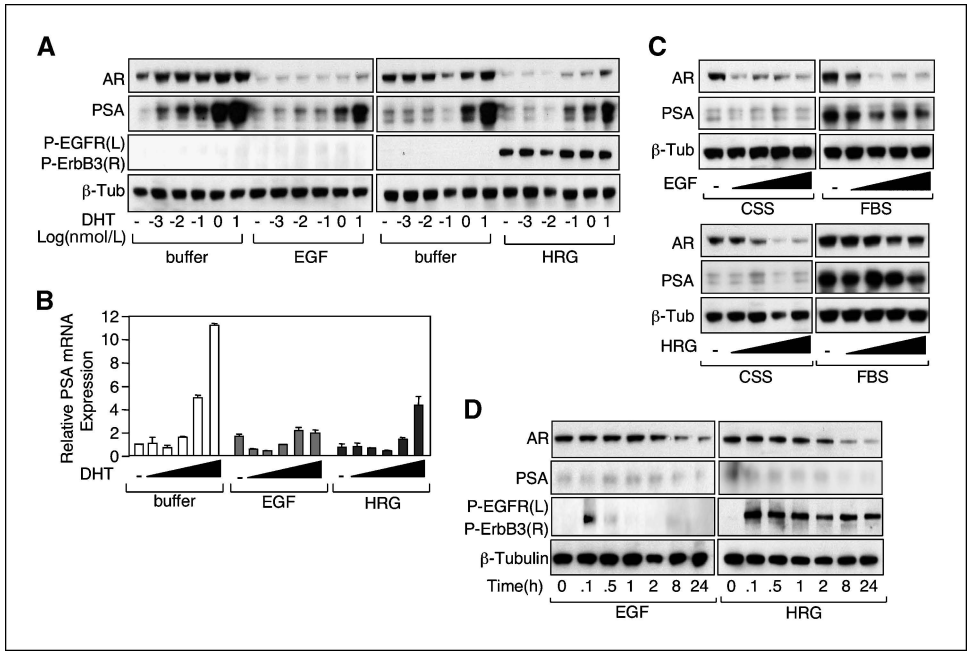

Figure 1. ErbB signaling decreases AR protein expression and transcriptional activity in LNCaP cells. LNCaP cells in 5% CSS medium were treated with 0, 10^{-3}, 10^{-2}, 10^{-1}, 1, or 10 nmol/L DHT in the absence or presence of 20 ng/mL EGF or 40 ng/mL heregulin-β1 for 24 h. *A,* equal amounts of total protein were immunoblotted for AR, PSA, phosphorylated EGFR (*P-EGFR*; Tyr^{845}) or phosphorylated ErbB3 (*P-ErbB3*; Tyr^{1289}). *B,* equal amounts of RNA were analyzed for PSA mRNA, with results normalized to an 18S RNA internal control. *C,* LNCaP cells, in either 5% CSS or 5% FBS medium, were treated with EGF or heregulin-β1 (0, 20, 40, 100, or 200 ng/mL) for 24 h and extracted proteins were then immunoblotted for AR or PSA expression. *D,* LNCaP cells in 5% CSS medium were treated with EGF or heregulin-β1 for 0, 0.1, 0.5, 2, 8, or 24 h and then immunoblotted for AR, PSA, phosphorylated EGFR (Tyr^{845}) or phosphorylated ErbB3 (Tyr^{1289}) expression. β-Tubulin was used as loading control.

by coamplification of 18S or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA.

Immunoblotting. Cell extracts were prepared by boiling for 15 min in 2% SDS buffer. Blots were probed with anti-PSA (1:3,000, polyclonal, BioDesign), anti-AR (1:2,000, polyclonal, Upstate), anti-FLAG (1:3,000, monoclonal, Sigma), anti-EGFR (1:1,000, polyclonal, Cell Signaling), anti–phosphorylated EGFR (Tyr^{845}; 1:1,000, polyclonal, Cell Signaling), anti–phosphorylated ErbB3 (Tyr^{1289}; 1:1,000, polyclonal, Cell Signaling), anti–phosphorylated AKT (Ser^{473}; 1:1,000, polyclonal, Cell Signaling), anti–phosphorylated ERK (Thr^{202}/Tyr^{204}; 1:1,000, polyclonal, Cell Signaling), anti–β-tubulin (1:2,000, monoclonal, Chemicon), or anti–β-actin (1:5,000, monoclonal, Abcom). Blots were developed with 1:5,000 antirabbit or antimouse secondary antibodies (Promega).

Results

ErbB signaling decreases endogenous AR protein expression and represses AR transcriptional activity in LNCaP cells. EGFR and ErbB2 signaling have been shown to increase AR activity, but most work has been done on transfected AR or using inhibitors, and it is unclear whether activation of ErbB receptors increases endogenous AR activity in PCa cells. As expected, AR protein and activity in LNCaP cells were significantly induced by DHT, based on increased expression of androgen-regulated PSA (Fig. 1*A*). In contrast, EGFR and ErbB2 activation with EGF and heregulin-β1, respectively, markedly suppressed PSA induction by DHT (Fig. 1*A, left* and *right*, respectively). Moreover, AR protein in the absence or presence of DHT was greatly reduced by EGF or heregulin-β1 (Fig. 1*A*). The activation of ErbB2 by heregulin-β1 was confirmed based on phosphorylation of ErbB3 (Fig. 1*A, right*). EGFR phosphorylation was not seen after 24 h of EGF treatment (Fig. 1*A, left*), consistent with its known rapid degradation after activation (see Fig. 1*D*). Confirming that EGF and heregulin-β1 were suppressing PSA transcription, androgen-induced PSA mRNA was markedly decreased by EGF and heregulin-β1 (Fig. 1*B*). These results indicated that activation of EGFR and ErbB2 were decreasing AR protein expression, leading to decreased AR activity (although both growth factors could stimulate proliferation in the absence or presence of androgen; data not shown).

To support this hypothesis, we next examined a range of EGF and heregulin-β1 concentrations. EGF at 20 ng/mL, which maximally stimulated EGFR activation (data not shown), markedly decreased AR protein at 24 hours in hormone-depleted medium (Fig. 1*C, left*) or in FBS medium (Fig. 1*C, right*), with a corresponding decrease in PSA protein. Heregulin-β1 similarly decreased AR expression, with the concentration required for maximal ErbB2 activation (40 ng/mL based on ErbB3 phosphorylation, data not shown) being consistent with the concentration that decreased AR and PSA protein (Fig. 1*C*). In time course experiments, EGFR activation (based on Tyr^{845} phosphorylation) could be detected after 0.1 hour but not at later times due to receptor down-regulation (Fig. 1*D, left*; data not shown). Robust ErbB3 phosphorylation was similarly detected at 0.1 hour but persisted for 24 hours (Fig. 1*D, right*). AR protein levels started to decline at ~2 hours, markedly decreased at 8 hours, and remained low after 24 hours.

ErbB signaling decreases AR in other PCa cell lines. To determine whether this repression of AR is LNCaP cell specific, we tested additional cells. LAPC4 cells have a wild-type AR and their growth is stimulated by androgen, but *in vitro* they express minimal PSA. Both EGF and heregulin-β1 in these cells slightly decreased the low levels of AR detected in the absence of DHT and greatly reduced AR in the presence of DHT (Fig. 2*A*). C4-2 cells were derived from a LNCaP xenograft that relapsed after castration, and the cultured cells have substantial AR activity (as assessed by PSA expression) in steroid hormone–depleted medium. EGF and heregulin-β1 both markedly decreased AR protein levels in these cells, and heregulin-β1 also suppressed PSA expression in response to DHT stimulation (Fig. 2*B*). Interestingly, PSA protein was decreased by EGF in the absence of exogenous DHT but was increased by EGF at 1 and 10 nmol/L DHT despite lower AR protein levels, possibly reflecting a marked increase in the activity of a coactivator in these cells (Fig. 2*B*). The AR in CWR22Rv1 cells has a point mutation and a duplicated exon 3, and these cells do not produce substantial PSA. AR protein in these cells could be increased by

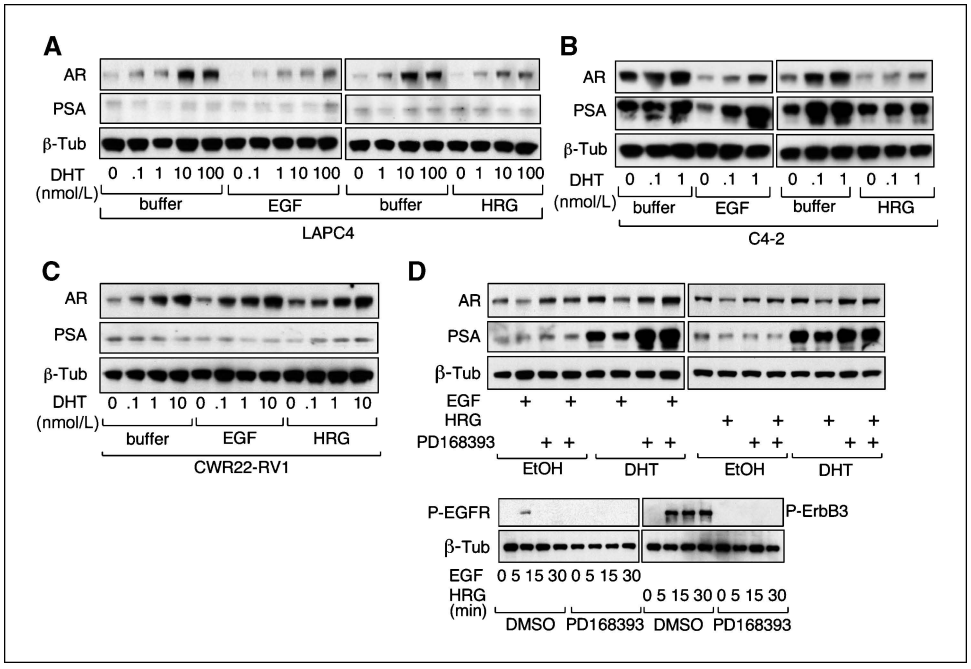

Figure 2. Effects of ErbB signaling on AR in other PCa cell lines. *A–C,* LAPC4, C4-2, or CWR22Rv1 cells in 5% CSS medium were treated with DHT (0–100 nmol/L) in the absence or presence of EGF or heregulin-β1 for 24 h, and equal amounts of protein were then immunoblotted for AR and PSA protein expression. *D, bottom,* LNCaP cells in 5% CSS medium were treated with EGF or heregulin-β1 in the absence or presence of PD168393 (10 μmol/L) for 0, 5, 15, or 30 min and then immunoblotted for phosphorylated EGFR (*P-EGFR*; Tyr^{845}) or phosphorylated ErbB3 (*P-ErbB3*; Tyr^{1289}) expression; *top,* LNCaP cells were treated with different combinations of PD168393, ethanol vehicle (0.1%), DHT (10 nmol/L), EGF (20 ng/mL), or heregulin-β1 (40 ng/mL) for 24 h and then immunoblotted for AR or PSA expression.

DHT, but EGF and heregulin-β1 had no clear effect on AR protein (Fig. 2*C*).

As expected, the irreversible EGFR/ErbB2 inhibitor PD168393 effectively blocked both EGFR ($pTyr^{845}$) and ErbB3 ($pTyr^{1289}$) activation in response to EGF and heregulin-β1, respectively (Fig. 2*D, bottom*). Moreover, EGF- and heregulin-β1–mediated repression of AR expression in LNCaP cells was abrogated by PD168393 (Fig. 2*D, top*). Interestingly, PD168393 increased androgen-induced PSA expression in the absence of growth factor stimulation, possibly due to the inhibition of basal EGFR or ErbB2 activity. Collectively, these data show that EGF, as well as heregulin-β1, markedly decrease both unliganded and liganded AR protein expression in several (but not all) AR- positive PCa cells.

ErbB signaling does not decrease expression of transfected AR. The results above are in contrast to some previous results with transfected AR (15, 18). Therefore, we used a triple-Flag tagged AR cDNA driven by a cytomegalovirus promoter to examine transfected AR in LNCaP and HeLa cells. In contrast to the above results with endogenous AR in LNCaP cells, EGF dramatically increased transiently transfected Flag-AR protein expression in the absence or presence of DHT (Fig. 3*A*). Heregulin-β1 also enhanced AR expression, but to a lesser extent than EGF. Similar results were obtained in HeLa cells (Fig. 3*B*). Because

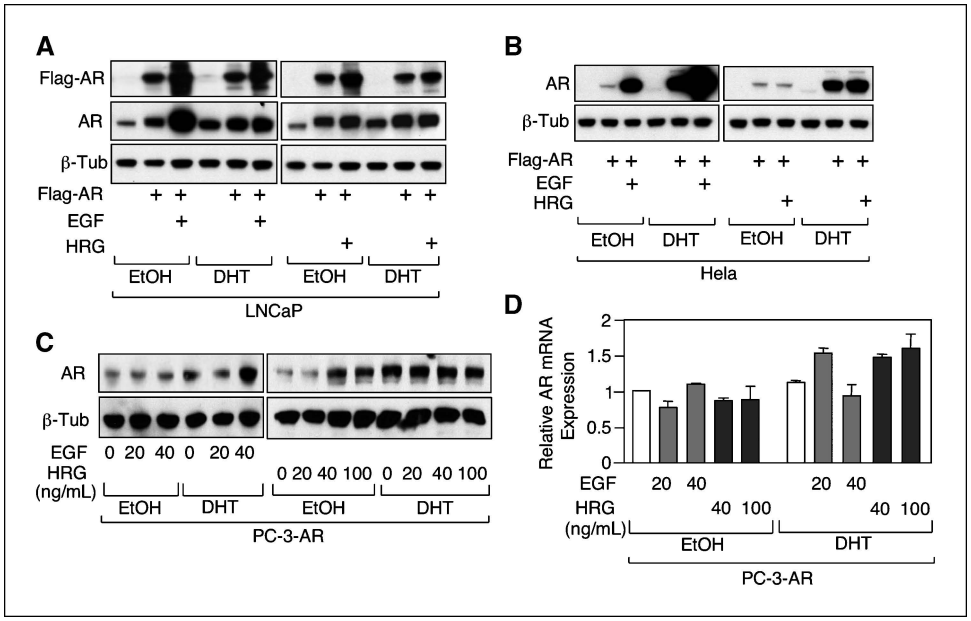

Figure 3. ErbB signaling does not decrease expression of transfected AR. *A,* LNCaP cells in 5% CSS medium were transfected with 0.25 μg Flag-AR for 24 h and then treated with EGF or heregulin-β1 in the absence or presence of DHT (10 nmol/L) for 24 h, and equal amounts of extracted proteins were immunoblotted for Flag (transfected AR) or total AR protein expression. *B,* HeLa cells in 5% CSS medium were transfected with 0.25 μg Flag-AR for 24 h, then treated with EGF or heregulin-β1 in the absence or presence of DHT (10 nmol/L) for 24 h, and immunoblotted for AR protein expression. *C,* PC-3 cells that stably express transfected AR (*PC-3-AR*) were grown in 5% CSS medium for 2 d, then treated with different concentration of EGF or heregulin-β1 in absence or presence of DHT (10 nmol/L) for 24 h, and immunoblotted for AR. β-Tubulin was used as loading control. *D,* AR mRNA expression in PC-3-AR cells treated as indicated for 24 h.

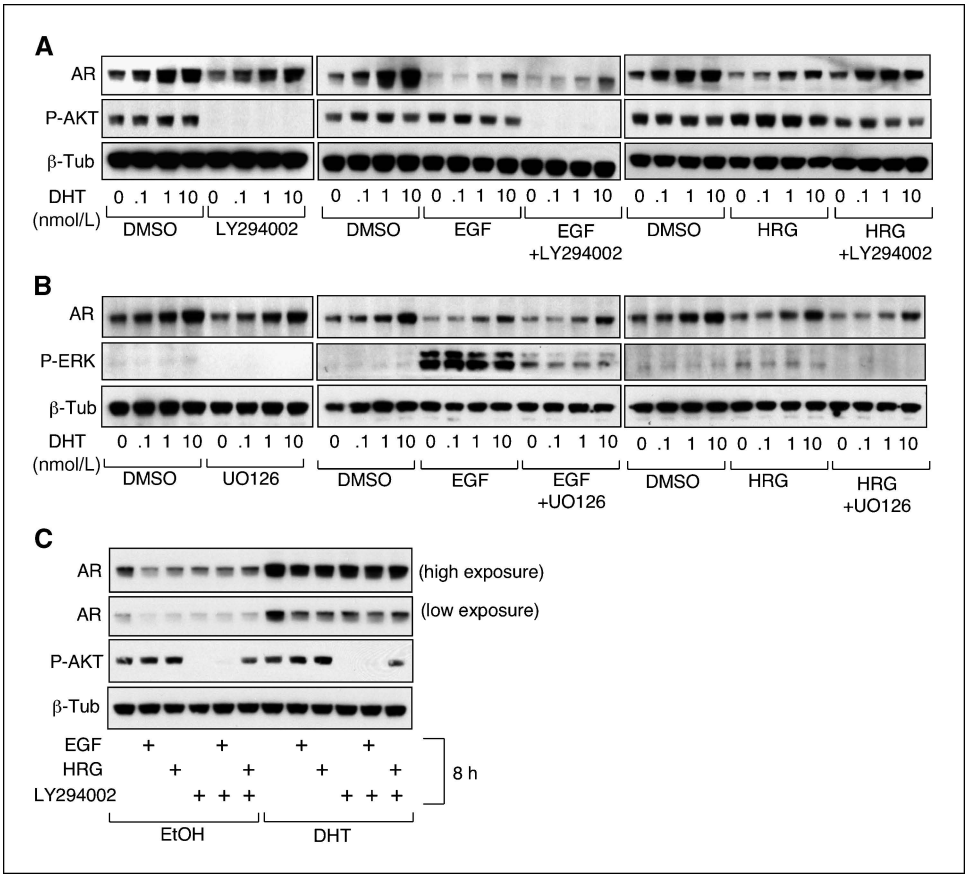

Figure 4. Contributions of PI3K and Erk to AR down-regulation by EGF and heregulin-β1. *A* and *B,* LNCaP cells in 5% CSS medium were treated with DHT (0-10 nmol/L), EGF (20 ng/mL), or heregulin-β1 (40 ng/mL), minus or plus LY294002 (40 μmol/L; *A*) or UO126 (10 μmol/L; *B*), as indicated for 24 h. Equal amounts of protein extracts were then immunoblotted for AR, phosphorylated AKT (*P-AKT*; Ser^{473}), or phosphorylated ERK (*P-ERK*; Thr^{202}/Tyr^{204}), with β-tubulin as a loading control. Cells receiving LY294002 or UO126 were pretreated with these inhibitors for 30 min before adding EGF or heregulin-β1. *C,* LNCaP cells were treated as above and analyzed by immunoblotting after 8 h.

transiently transfected cells express high levels of AR protein that may not be regulated by physiologic mechanisms, we also examined PC3 cells (an AR-negative PCa cell line) that were stably transfected with the AR expression vector. AR expression in these cells was modestly increased by EGF, and expression in the absence of DHT was markedly increased by heregulin-β1 (Fig. 3*C*). Significantly, AR mRNA levels in the PC3-AR cells were not markedly altered by these growth factors, indicating that AR protein translation or stability were being increased (Fig. 3*D*). In any case, as these data showed that endogenous versus transfected AR respond differently to ErbB pathway activation, we continued to focus on mechanisms regulating endogenous AR expression.

EGF decreases AR expression independently of PI3K and Erk activation, whereas PI3K contributes to AR down-regulation by heregulin-β1. We next examined whether the PI3K/Akt or Ras/Raf/Erk pathways, both of which can modulate AR function, were required for the EGF- or heregulin-β1–induced decrease in AR expression. LNCaP cells are PTEN deficient, so PI3K pathway activation is evidenced by high basal phosphorylated Akt, which was further enhanced by EGF (Fig. 4*A, left and middle*). Heregulin-β1 more strongly increased phosphorylated Akt levels, reflecting the robust recruitment and activation of PI3K by phosphorylated ErbB3 (Fig. 4*A, right*). The PI3K inhibitor LY294002 completely blocked the basal and EGF-stimulated Akt phosphorylation in LNCaP cells but did not prevent the marked decrease in AR protein in response to EGF (Fig. 4*A, left and middle*). In contrast, LY294002 substantially prevented the decrease in AR protein by heregulin-β1, despite only partially suppressing PI3K activation based on Akt phosphorylation (Fig. 4*A, right*).

Whereas EGF did not markedly enhanced PI3K activity in LNCaP cells, it very strongly activated the Ras/Raf/Erk pathway as evidenced by immunoblotting for phosphorylated Erk1/2 (Fig. 4*B, middle*). The MEK inhibitor UO126 blocked Erk activation in response to EGF but did not prevent the decrease in AR, indicating that EGF is not suppressing AR expression through Erk activation (Fig. 4*B, middle*). Heregulin-β1 only weakly stimulated Erk, and UO126 similarly did not block its ability to decrease AR expression (Fig. 4*B, right*).

As the above experiments examined AR after 24 hours, we next examined whether PI3K was contributing to the rapid decline in AR protein that can be clearly observed by 8 hours. Significantly, LY294002 did not prevent the marked decline in AR protein mediated by EGF or heregulin-β1 at 8 hours (Fig. 4*C*). We conclude that PI3K contributes to the decline in AR protein at 24 hours but that a distinct PI3K independent mechanism is mediating the rapid decline in AR protein between 2 and 8 hours in response to EGF and heregulin-β1.

AR protein degradation is not increased by EGF or heregulin-β1. To determine whether EGF or heregulin-β1 were increasing AR degradation, we used cycloheximide to inhibit new protein synthesis and assess AR protein stability. Cells in steroid-depleted medium (minus or plus DHT) were treated with cycloheximide alone or in conjunction with EGF or heregulin-β1, which were added 2 hours before the cycloheximide. This 2-hour pretreatment with growth factors was selected as AR protein expression is starting to decline at this time, and longer pretreatment results in much

lower baseline levels of AR that make half-life comparisons problematic. However, it should be noted that effects due to proteins that are induced by androgen after 2 hours may be missed. Cells were harvested at time 0 (immediately before cycloheximide addition) and at 4 to 24 hours. As seen in Fig. 5*A*, neither EGF nor heregulin-β1 substantially increased the rate of AR protein degradation at up to 8 hours, although degradation at 24 hours was increased. These results indicate that increased AR protein degradation does not account for the decline in AR protein levels that are observed within 8 hours of EGF or heregulin-β1 (see Fig. 1*D*) but may contribute to a further decline at later times.

EGF and heregulin-β1 increase degradation of AR mRNA. As AR protein degradation was not markedly increased by EGF or heregulin-β1 after up to 8 hours, we next assessed effects on AR mRNA. EGF markedly decreased endogenous AR mRNA by up to ~80% at 24 hours, whereas heregulin-β1 decreased AR mRNA by ~60% (Fig. 5*B, left*). These decreases occurred in the absence or presence of androgen. Moreover, they were observed within 4 hours, consistent with the rapid decline in AR protein (Fig. 5*B, right*). Significantly, AR mRNA levels were decreased by EGF and heregulin-β1 over a broad range of DHT concentrations, indicating that these growth factors are overriding mechanisms that enhance AR mRNA expression in response to androgen deprivation and low AR protein levels (37).

A regulatory element that represses AR gene transcription has been identified in the 5′ untranslated region (UTR), and it has been reported that a complex of Purα and hnRNPk binds this element and represses AR mRNA transcription (38–42). However, we did not detect increased expression of Purα or hnRNPk in response to EGF or heregulin-β1 (data not shown). Although this did not rule out posttranslational modifications in Purα or hnRNPk or decreased AR transcription by other mechanisms, we next examined AR mRNA stability. LNCaP cells (grown in medium minus or plus DHT) were pretreated with growth factors or vehicle for 8 hours, and actinomycin D was then added to block the new mRNA synthesis. In the absence of DHT or growth factors, AR mRNA had a half-life of ~8 hours, which was substantially decreased to ~4 hours in the presence of EGF or heregulin-β1 (Fig. 5*C, left*). EGF and heregulin-β1 similarly decreased AR mRNA half-life in the presence of DHT (Fig. 5*C, right*). It should be noted that the rate of AR mRNA degradation in the untreated cells increases abruptly after ~4 hours, which may

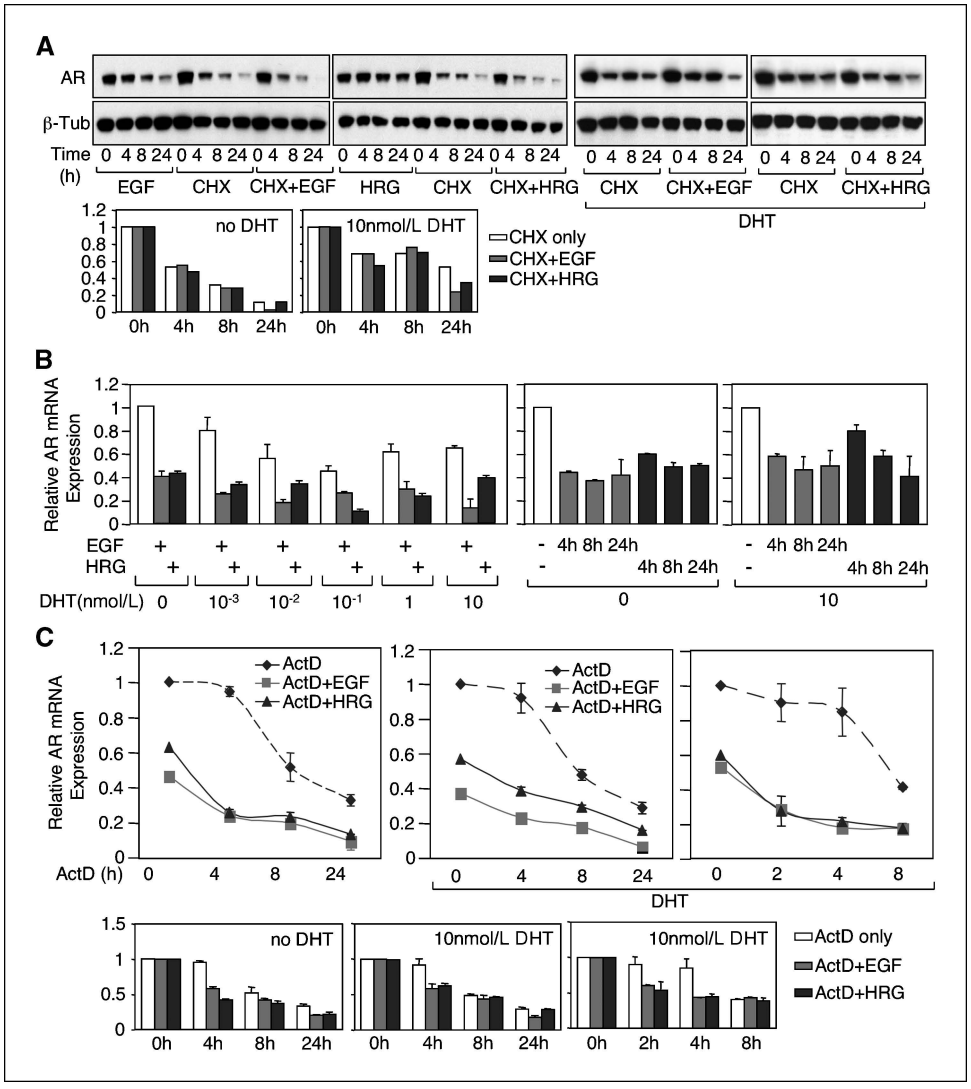

Figure 5. EGF and heregulin-β1 decrease AR mRNA levels and increase AR mRNA degradation. *A,* LNCaP cells in 5% CSS medium (*left*) or in 5% CSS medium with 10 nmol/L DHT (*right*) were treated with cycloheximide (10 ng/mL), EGF (20 ng/mL), and/or heregulin-β1 (40 ng/mL), as indicated, and then immunoblotted for AR. Cells were pretreated with EGF or heregulin-β1 at 2 h before cycloheximide treatment. *Bottom,* quantified results. *B,* LNCaP cells in 5% CSS were treated for 24 h (*left*) or over a 0- to 24-h time course (*right*) with 0 to 10 nmol/L DHT, minus or plus EGF (20 ng/mL) or heregulin-β1 (40 ng/mL). AR mRNA normalized to 18S RNA. *C,* LNCaP cells in 5% CSS medium (*left*) or in 5% CSS medium with 10 nmol/L DHT (*right*) were treated with actinomycin D (10 μmol/L), minus or plus EGF (20 ng/mL) or heregulin-β1 (40 ng/mL) for 0, 2, 4, 8, or 24 (8 h pretreatment with growth factors, the 0–24 h and 0–8 h time courses with DHT are from separate experiments). Equal amounts of RNA were then analyzed for AR mRNA expression (normalized to GAPDH mRNA) by real-time RT-PCR. *Bottom,* quantified results, with all values at time 0 being normalized to 1.

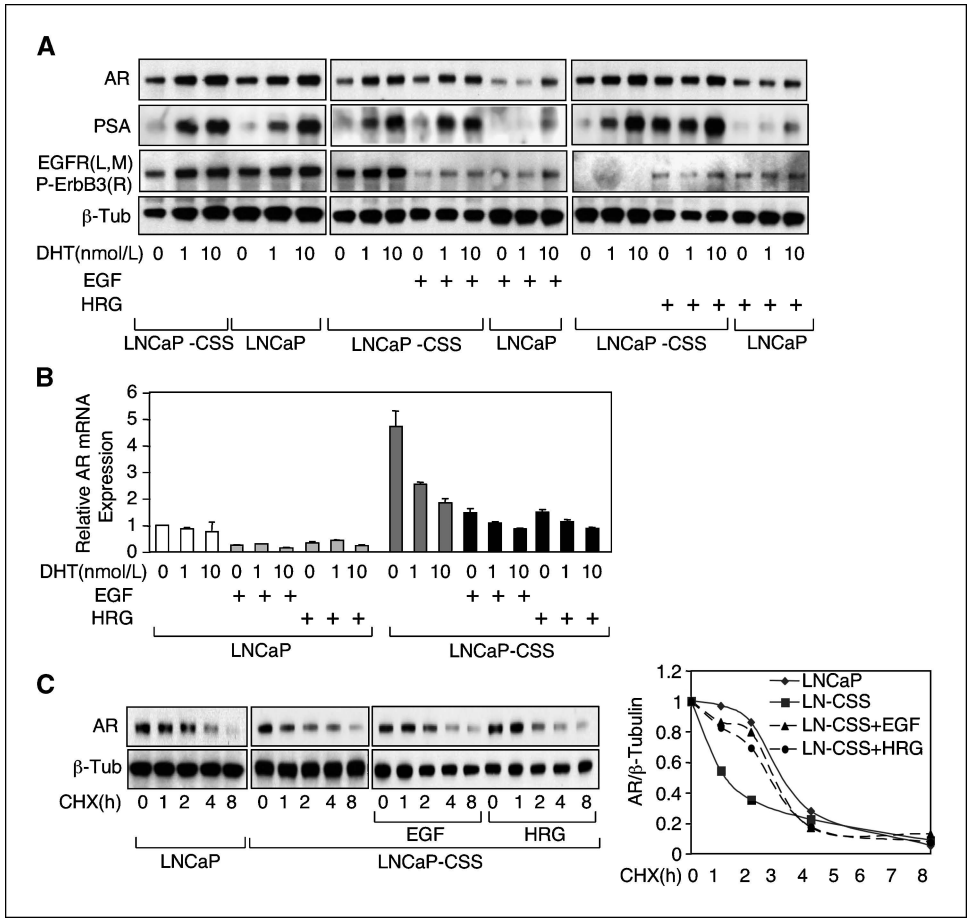

Figure 6. EGF and heregulin-β1 increase AR protein stability in LNCaP cells adapted to growth in androgen-depleted medium. *A* and *B,* LNCaP cells were cultured in either 10% CSS medium (*LNCaP-CSS*) or 10% FBS medium for ~4 to wk. The LNCaP-CSS and control LNCaP cells were then grown in 5% CSS medium for 2 d and then treated for 24 h with 0, 1, or 10 nmol/L DHT in the absence or presence of EGF (20 ng/mL) or heregulin-β1 (40 ng/mL). *A,* equal amounts of protein were immunoblotted for AR, PSA, EGFR (*left*), or phosphorylated ErbB3 (*P-ErbB3*; Tyr^{1289}; *right*) expression. *B,* equal amounts of RNA were analyzed for AR mRNA by real-time RT-PCR (normalized using internal GAPDH control). *C,* control LNCaP and LNCaP-CSS cells in 5% CSS medium were treated with cycloheximide (10 ng/mL), minus or plus EGF or heregulin-β1, for 0, 1, 2, 4, or 8 h, and equal amounts of protein were then immunoblotted for AR. *Right,* quantification of AR normalized to β-tubulin.

reflect an actinomycin D–induced degradative pathway and result in an underestimation of AR mRNA stability in the untreated cells. In any case, the data indicate that increased mRNA degradation contributes to the decline in AR mRNA in response to EGF and heregulin-β1.

EGF and heregulin-β1 increase AR protein stability in LNCaP cells adapted to growth in androgen-depleted medium. Studies using patient samples and xenograft models have shown that AR mRNA levels are high in CRPC and are increased relative to primary untreated PCa (4, 43–45). Therefore, as EGFR and ErbB2 activities may be increased in CRPC, we considered whether EGF and heregulin-β1 would still suppress AR mRNA levels in PCa cells adapted to grow under androgen-deprived conditions. To test this hypothesis, we changed the growing condition of LNCaP cells from medium with normal FBS to medium with steroid-depleted CSS.

Short-term culturing (1 week) in CSS medium did not significantly affect the suppression of AR protein by EGF or heregulin-β1 (data not shown), but a longer-term culture (~4–6 weeks) in CSS medium did alter this response. As shown in Fig. 6*A*, AR protein levels in the LNCaP-CSS cells (cells grown in CSS medium for ~4–6 weeks), in the absence or presence of DHT, were not decreased by EGF or heregulin-β1 (Fig. 6*A*). Immunoblotting for EGFR (which is rapidly down-regulated in response to activation) and pErbB3 confirmed that both the LNCaP and LNCaP-CSS cells were stimulated by EGF and heregulin-β1. Interestingly, in the LNCaP-CSS cells, heregulin-β1 stimulated the expression of PSA in the absence of added DHT (Fig. 6*A, right*), consistent with the conclusion that ErbB2 stimulation can, under some conditions, enhance AR transcriptional activity in the absence of androgens or at low androgen levels (6, 23–25).

Significantly, AR mRNA levels were markedly increased in the LNCaP-CSS versus the parental LNCaP cells and rapidly declined in response to DHT (Fig. 6*B*). However, although EGF and heregulin-β1 did not decrease AR protein levels in the LNCaP-CSS cells, they both still markedly decreased AR mRNA levels in the absence and presence of DHT (Fig. 6*B*). Therefore, as these growth factors were still decreasing AR mRNA but not AR protein, we examined AR protein stability in the LNCaP versus LNCaP-CSS cells (pretreated for 2 hours with EGF or heregulin-β1 before addition of cycloheximide at time 0). AR protein was less stable (half-life ~1 hour) in the LNCaP-CSS cells grown in CSS medium than in the parental LNCaP cells in the same medium (half-life ~2.0 hours; Fig. 6*C*), indicating that the LNCaP-CSS cells adapted to androgen deprivation primarily by increasing AR mRNA levels. However, in contrast to the parental LNCaP cells (see above), both EGF and heregulin-β1 increased AR protein half-life in LNCaP-CSS cells from ~1 to ~3 hours (Fig. 6*C*, quantified in the *right*). This result indicates that increasing AR protein stability through activation of EGFR or ErbB2 is a mechanism that may contribute to maintaining AR protein expression in CRPC, particularly if it can become uncoupled from the down-regulation of AR mRNA.

Discussion

Previous studies indicate that stimulation of EGFR and ErbB2 can enhance AR stability and transcriptional function and may contribute to AR activity in CRPC (6, 15, 18, 22–25, 27). We initially examined LNCaP PCa cells to further define the molecular basis for these effects on AR and found that stimulation with both EGF and heregulin-β1 rapidly decreased expression of AR protein and the androgen-regulated *PSA* gene over a broad range of DHT concentrations. This decrease in AR protein was also observed in LAPC4 and C4-2 cells but not in CWR22Rv1 cells. Consistent with the latter result, AR protein in another CWR22-derived cell line (CWR-R1) was not changed in response to EGF or heregulin (18, 23). The rapid AR down-regulation in response to EGF and heregulin-β1 was not prevented by UO126 or LY294002, indicating that it was not mediated through the Erk or PI3K pathways. Moreover, AR protein degradation was not rapidly enhanced by EGF or heregulin-β1. In contrast, AR mRNA levels were rapidly decreased by both EGF and heregulin-β1 over a broad range of DHT concentrations. Decreased AR transcription likely contributes to this decrease, but AR mRNA degradation was also increased in response to EGF and heregulin-β1. Taken together these findings show that EGFR and ErbB2 activation, while having multiple effects on AR activity through diverse mechanisms, markedly decrease AR mRNA expression and increase AR mRNA degradation.

The AR has a long 3′ UTR, which contains a highly conserved UC-rich region implicated in the regulation of mRNA stability (46). Therefore, EGFR or ErbB2 may regulate expression of RNA binding proteins that interact with this UC-rich region (47). Decreased AR mRNA transcription also likely contributes to the marked decrease in AR mRNA levels in response to EGF and heregulin-β1. AR transcription may be regulated by multiple factors, including a suppressor element in the AR 5′ UTR (40, 48–52). Further studies are clearly needed to define the precise mechanisms by which EGF and heregulin-β1 are enhancing AR mRNA degradation and to assess their effects on AR mRNA transcription.

Previous studies indicated that EGF could enhance AR activity and that ErbB2 could enhance AR stability and responses to low levels of androgen (6, 15–18, 23–27). However, other studies in LNCaP cells found that EGF or HB-EGF decrease AR expression, consistent with the findings in the current study (28–31). One factor that may contribute to differences between studies is that results in some cases are based on transfected AR (15, 18). Another factor is the use of EGFR/ErbB2 inhibitors in some studies to examine the effects of basal growth factor receptor activity on the endogenous AR versus the use of EGF and heregulin-β1 to examine the response to EGFR/ErbB2 activation in the current study (22, 24, 25). Whereas one might conclude that decreased AR activity in response to EGFR/ErbB2 inhibitors would predict increased AR activity in response to EGF and heregulin-β1, this may not be the case as the rapid high-level stimulation with EGF/heregulin-β1 may be eliciting distinct responses. Therefore, whereas the results in this study identify a novel mechanism by which EGFR and ErbB2 can suppress AR expression, the overall response to activation or inhibition of these receptors *in vivo* may be variable and not readily predictable due to interactions between multiple downstream pathways.

As noted above, EGFR and ErbB2 activate multiple downstream pathways that may directly or indirectly modulate AR expression and function. One example in this study was that EGF treatment caused a strong increase in DHT-stimulated PSA expression in C4-2 cells despite a decrease in AR protein. This is consistent with a previous study showing that EGF can increase phosphorylation and activity of the p160 transcriptional coactivator SRC-2/TIF2/GRIP1 (18). A second example was the ability of the PI3K inhibitor LY294002 to partially block the heregulin-β1–stimulated decline in AR protein at 24 hours (but not 8 hours), which is consistent with a previous study showing that mTOR activation in response to HB-EGF caused a decrease in AR translation (30). A third example is that heregulin-β1 increased AR protein stability and stimulated PSA expression in the LNCaP-CSS cells in the absence of added DHT. These effects are similar to those observed in LAPC4 cells adapted to grow under castrate conditions, although their molecular basis remains to be defined (24). Importantly, the LNCaP-CSS cells also adapted to androgen deprivation by increasing their AR mRNA to maintain AR protein levels. However, heregulin-β1 still markedly decreased AR mRNA levels in these cells so that heregulin-β1 did not increase AR protein levels. It will be important to determine in CRPC patients whether the mechanisms that decrease AR mRNA in response to EGF/heregulin-β1 are uncoupled from mechanisms that enhance AR transcriptional activity and to determine whether these former mechanisms can be targeted by drugs to prevent the increase in AR mRNA levels that occurs in CRPC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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